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Microheterogeneity of parsley plastocyanin

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A procedure for isolation of two iso-plastocyanins from parsley has been described here. Three consecutive chromatographic steps on DE-52-Whatman cellulose were applied for isolation of two total plastocyanin (PC) fractions, oxidized [PC(II)] and reduced [PC(I)]. By chromatofocusing of PC(II) on Polybuffer exchanger 74 two different plastocyanins, designated as plastocyanin *a* (PC*a*) and plastocyanin *b* (PC*b*), were obtained. The isoelectric points (pI) of PC*a* and PC*b* at 10°C are 4.16 and 4.14, respectively. The complete amino acid sequences of PC*a* and PC*b* were determined. The two iso-proteins consist of 97 amino acid residues and differ only at sequence position 53, where Glu in PC*a* is replaced by Asp in PC*b*.

Primary structure; Plastocyanin *a*; Plastocyanin *b*; Microheterogeneity; Parsley

1. INTRODUCTION

Plastocyanin is a metalloprotein of 10,5 kDa, containing a blue (type I) cooper. Its functional role is that of an electron mediator between cytochrome b_6/f complex and photosystem I [1–3]. Plastocyanin is considered to be weakly linked to the thylakoid membrane [4] and/or freely diffusible in the lumen [5].

Plastocyanin from about 70 plants has been isolated. Some structural and physicochemical and functional characteristics of this protein are mentioned elsewhere [6–9]. The plastocyanin primary structure has been completely determined for one blue-green alga, *Anabaena variabilis* [10], four green algae, *Chlorella fusca* [11], *Enteromorpha prolifera* [12], *Scenedesmus obliquus* (Kelly, J.M. and Ambler R.P., unpublished work cited in [8]), *Ulva arasakii* [13] and 18 higher plants ([14] and references cited there). The amino acid residue contents of the analyzed structures vary from 97 to 105. The comparative analysis of the plastocyanin primary structures reveals two opposite trends. On the one hand – the set of invariant or highly conservative residues that, in case of higher plants, exceed the half of their total number. That is particularly relevant to the 31–45 and 82–94 residues. They involve the invariant His-37, Cys-84, His-87 and Met-92, supplying electrons in the coordinate sphere of the Cu atom, the conservative or invariant Ala-33, Phe-35, Pro-36, Ile/Val-39, Pro-86, Ala-90, located in the "north" hydrophobic pocket and 42–44 residues, included in the "east" acidic patch [8, 15]. The other functionally less significant molecular regions, however, display considerable variability of species. Comparisons have shown that plastocyanin is evolving at least twice as fast as cytochrome *c* of plants [16]. Plastocyanin amino acid sequences data have been used in phylogenetic classification at the familial and tribal levels [16–18]. To this end both complete and partial sequences (40 N-terminal residues) of plastocyanin from some members of 11 families of flowering plants have also been determined. In more than 20 species two amino acid residues occur in some positions. In these cases the reasons for heterogeneity remain unknown. "Whilst the bulk purification procedures used did not separate different forms of plastocyanins, the presence of 1–4 protein components, depending on the taxa, were demonstrated by iso-focusing of plastocyanin preparations on analytical gels" [17].

Two different plastocyanins, designated as PC*a* and PC*b*, have been isolated and characterized in poplar, *Populus nigra*, var. *Italica* [19]. It was found [20] that PC*a* is related to the well characterized about primary (Ambler, R.P., unpublished work, cited in [21]) and three-dimensional structure [15, 21] poplar plastocyanin. PC*b* sequence differs from PC*a* at 12 positions.

This paper reports a procedure for isolation of PC*a* and PC*b* from parsley and the complete amino acid sequences of the two iso-plastocyanins.

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Abbreviations: T, tryptic peptides of PC*a* and PC*b*; S*a* and S*b*, Staphylococcal protease peptides of PC*a* and PC*b*, respectively

2. MATERIALS AND METHODS

2.1. *Materials*

PC*a* and PC*b* were prepared from commercially obtained parsley,

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var. *'Festival'*. DE-52 cellulose was obtained from Whatman, Polybuffer exchanger (PBE) 94 and Polybuffer 74 were supplied from Pharmacia. Trypsin (Sigma) and *Staphylococcus aureus* V8 (Miles Laboratories) were used without additional treatment. Reagents used for high performance liquid chromatography (HPLC) and amino acid sequence analysis were supplied by Applied Biosystems Inc. (Foster City, CA, USA). Double-distilled water, additionally filtered through a Milli-Q system (Millipore), was used.

2.2. *Preparation of PCa and PCb*

The extraction and isolation of the total PC was performed according to [23], with some modifications. Chromatofocusing was applied for separation of PC into PC*a* and PC*b*. Fresh leaves (5 kg) were used as the starting material. PC was isolated from the total protein extract on the first chromatographic column (DE-52, 3.5×25 cm) by linear gradient elution with 0.06–0.5 M Tris-HCl buffer, pH 7.6, as a last fraction. PC was dialyzed against 0.02 M Na-phosphate buffer, pH 6.9, and purified on the second chromatographic column (DE-52, 2.6×20 cm), using 0.02–0.2 M linear gradient of the same buffer. The purified PC was dialyzed against 0.03 M Na-phosphate buffer, pH 6.9, oxidized by treatment with some crystals of $K_3[Fe(CN)_6]$ and applied on the third chromatographic column (DE-52, 1.6×30 cm), equilibrated with 0.03 M of the same buffer. With 0.03–0.15 M linear gradient of the buffer, from the column were eluted an oxidized plastocyanin [PC(II)], a reduced plastocyanin [PC(I)] and an unidentified protein fraction (P) (Fig. 1a). After concentrating and desalting by ultrafiltration through YM-5 Amicon membrane PC(II) and PC(I) were subjected to analytical isoelectric focusing (IEF) on Servalit pH 3–6 and LKB pH 4–5 (Fig. 1b) polyacrylamide gel (PAG) plates, using LKB 2117 Multiphor unit and LKB 2197 power supply. PC(II) (15–20 mg/ml) was dissolved in 5–10 ml 0.025 M piperazine-HCl buffer, pH 5.5, and applied on a fourth column with PBE 94 (0.9×30 cm), equilibrated with the same buffer. The elution was performed by Polybuffer 74, ten-fold diluted and titrated to pH 4.0 with conc. HCl. As a result two oxidized plastocyanin fractions, PC*a* and PC*b* (approximate ratio 1:1), were obtained (Fig. 2a). PC*a* and PC*b* were immediately titrated with 0.5 M Na_2HPO_4 to pH 6.9, desalted and concentrated by ultrafiltration and stored at −25°C. The protein purity was verified by SDS-polyacrylamide disk-electrophoresis (Fig. 2b). 4.5% T stacking gel and 15% T running gel (2 mm) were used; the electrode buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS.

2.3. *Structural analysis*

The complete amino acid sequences of PC*a* and PC*b* were determined by overlapping of their partial N-terminal sequences and two types of peptides. 10 nmol of PC*a* and PC*b* were digested with trypsin in 0.1 M NH_4HCO_3, pH 8.0, after blocking of the Cys SH group by 4-vinylpyridine [20]. Another 10 nmol of Cys-vinylpyridylated PC*a* and PC*b* were hydrolyzed with *Staphylococcus aureus* in the same buffer [24]. Peptides were separated by reversed-phase HPLC on a Gilson chromatographic system, using a 0.46×22-cm Ultrasphere ODS (Applied Biosystems) RP-300 column. The N-terminal groups of the peptides as well as of the whole PC*a* and PC*b* were determined by manual dansyl-Edman degradation, using Schleicher and Schuell polyamide plates. The amino acid compositions of the peptides and PC*a* and PC*b* were determined with Durrum D-500 amino acid analyzer after hydrolysis with 5.7 N HCl in vacuo at 110°C for 24 h. Automated sequence analysis of all peptides and the whole PC*a* and PC*b* was carried out on an Applied Biosystems model 470 A sequencer [20].

3. RESULTS

PC*a* and PC*b* were isolated from parsley leaves, grown in autumn. The molecular heterogeneity of plastocyanin, however, was confirmed by IEF of water-acetonic extract of different parsley varieties, grown in different regions notwithstanding the season.

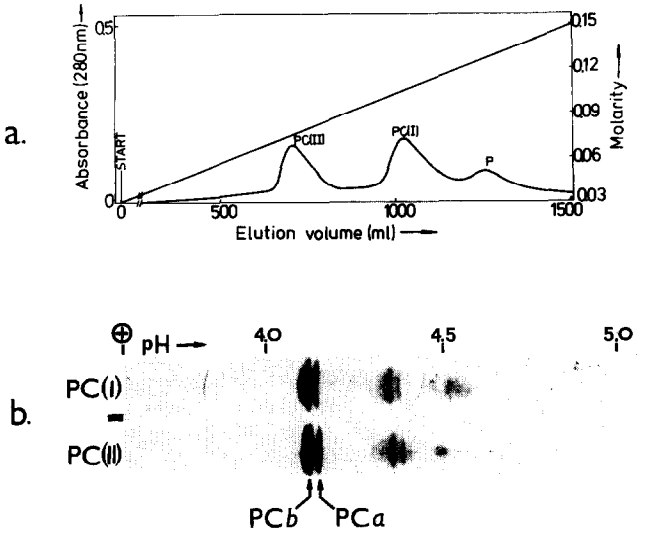

Fig. 1. (a) Separation of PC(II) and PC(I) by anion-exchange chromatography of PC on DE-52-Whatman. Linear gradient 0.03–0.15 M of sodium phosphate buffer, pH 6.9. Registration at 280 nm. (b) Isoelectric focusing of PC(II) and PC(I) on LKB pH 4–5 PAG plate at 10°C.

After three chromatographic steps for isolation and purification, three main fractions were obtained on the last DE-52 column (Fig. 1a). The absorption spectra (not shown), registered at 240–800 nm range, revealed the availability of two plastocyanin fractions, PC(II) and PC(I) and an another protein fraction (P), respectively. The IEF on LKB PAG plate, pH 4–5, displayed that both, PC(II) and PC(I), consisted of two major compounds with close pI-values, PC*a* (more alkaline) and PC*b* (more acidic), differing in their minor component contents (Fig. 1b). The pI-values of PC*a* and PC*b*

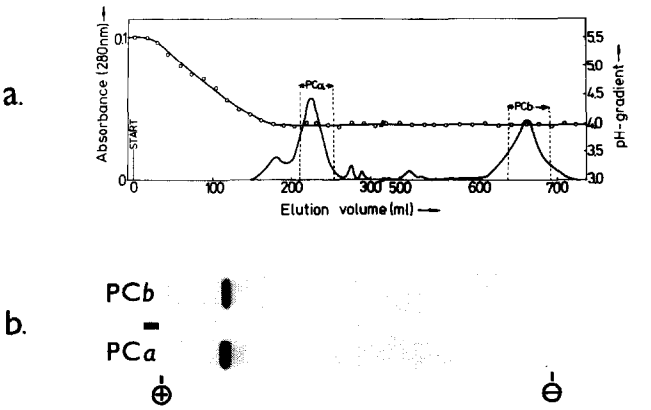

Fig. 2. (a) Separation of PC*a* and PC*b* by chromatofocusing of PC(II) on PBE 94. Start buffer, 0.025 M piperazine-HCl, pH 5.5. Eluent buffer, Polybuffer 74. Registration at 280 nm. Elution profile (——), left ordinate. pH-gradient (—○—○—), right ordinate. (b) SDS-PAG electrophoresis of PC*a* and PC*b*.

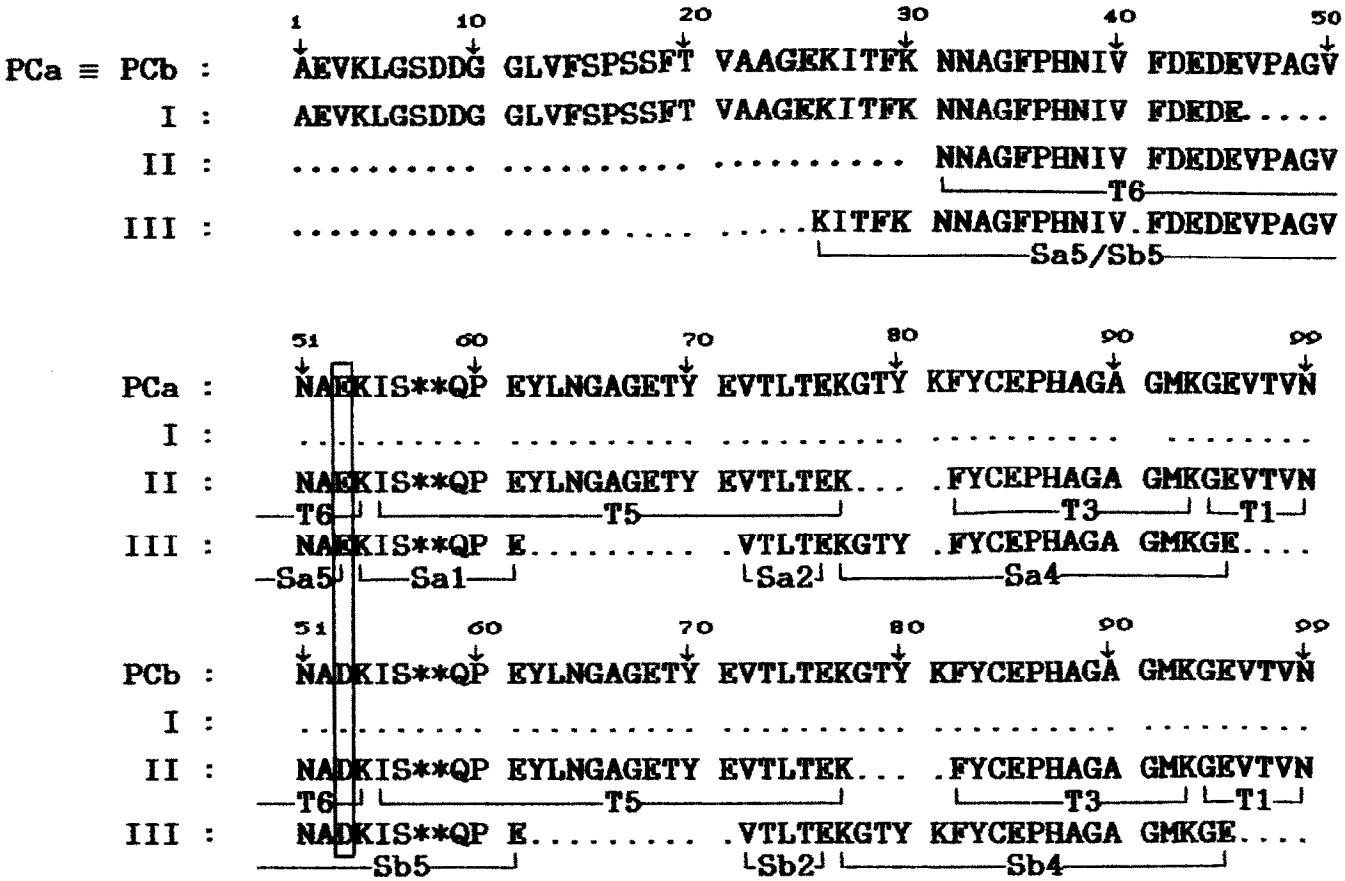

Fig. 3. The primary structures of parsley PC*a* and PC*b* and their peptide fragments. Ala-1-Val-50 fragments of PC*a* and PC*b* are identical (see text).

were determined on Serva PAG plates, pH 3–6 (not shown) toward Serva protein test mixture 9. The average pI-values of three independent measurements were found to be 4.16 and 4.14 pH-units for PC*a* and PC*b*, respectively (at 10°C).

The chromatofocusing of PC(II) by linear pH 5.5–4.0 gradient resulted in two oxidized plastocyanin fractions, PC*a* and PC*b* (Fig. 2a). These fractions were practically obtained at the isocratic (pH 4.0) elution conditions. The electrophoretic pattern of PC*a* and PC*b* proved their purity (Fig. 2b).

The absorption ratios A_{278}/A_{597} in 0.1 M Na-phosphate buffer, pH 6.9, were found to be 3.4 and 2.5 for PC*a* and PC*b*, respectively. Some subtle differences between the PC*a*- and PC*b*-absorption spectra observed in the near UV- and 500–800 nm areas could be accepted as essential (Donchev, A.A. and Dimitrov, M.I., unpublished).

By total sequencing of whole PC*a* and PC*b* the sequence of the first 45 amino acid residues was determined (Fig. 3, I). The two iso-proteins are identical in this region of the polypeptide chain. There were not any differences in the peptide maps obtained after trypsin hydrolysis of PC*a* and PC*b* (Fig. 4). Nine peptides, designated as T1, T2, T3, T4, T5, T6, T7, T8 and T9, were obtained. Their N-terminal and amino acid analyses, compared with the previously reported parsley plastocyanin structure [8] revealed that T1 was

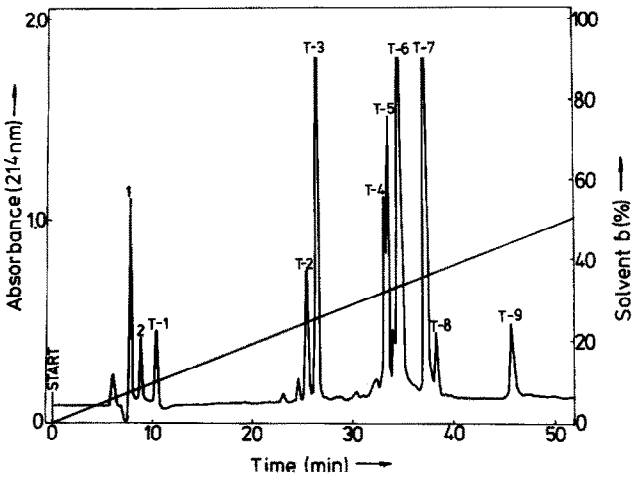

Fig. 4. HPLC of 0.1 mg trypsin hydrolysates of vinylpyridylated PC*a* and PC*b* (see the text). Linear gradient from 0 to 45% solvent B in 52 min. Flow rate, 0.5 ml/min; solvent A: 0.1% trifluoroacetic acid; solvent B: 0.1% trifluoroacetic acid in 100% acetonitrile. Registration at 214 nm. Peaks were characterized by N-terminal and amino acid analysis.

the C-terminal Gly-94-Asn-99 peptide, T2 was in line with Ile-27-Lys-30, T3 with Phe-82-Lys-93, T4 with Ile-55-Leu-63 (unspecific trypsin hydrolysis), T5 with Ile-55-Lys-77, T6 with Asn-31-Lys-54, T7 with Leu-5-Lys-26, T8 with Leu-5-Phe-19 (unspecific trypsin hydrolysis) and T9 was found to be non-hydrolyzed protein fraction (Fig. 3, II); the peaks 1 and 2 were of nonpeptide nature. The amino acid sequence analysis revealed that T1, T3 and T5 of PC*a* were identical with those of PC*b*; the only difference was found in position 53 of T6, where Glu in PC*a* was replaced by Asp in PC*b* (Fig. 3, II).

In order to overlap the Lys-54, Lys-77, Lys-81 and Lys-93 positions, *Staphylococcus aureus* hydrolysis of PC*a* and PC*b* was performed. The obtained two peptide maps, however, were not identical: Sa1, obtained at 21th minute of the elution time, was a PC*a*-derivative only (Fig. 5a); this peak was absent in the PC*b*-peptide map (Fig. 5b). The analyzed PC*a*-peptides Sa1, Sa2, Sa3, Sa4, Sa5 and Sa6 were assigned to correspond to residues Lys-54-Glu-61, Val-72-Glu-76, Tyr-62-Glu-71, Lys-77-Glu-95, Lys-26-Glu-53 and Val-3-Glu-25, respectively (Fig. 3, PC*a*, III). On the other hand, the PC*b*-peptides Sb2, Sb3, Sb4, Sb5 and Sb6 corresponded to residues Val-72-Glu-76, Tyr-62-Glu-71, Lys-77-Glu-95, Lys-26-Glu-61 and Val-3-Glu-25, respectively (Fig. 3, PC*b*, III). According to expectation Sa2, Sa3, Sa4 and Sa6 were identical with Sb2, Sb3, Sb4 and Sb6, respectively. Sa5, however, was shorter than Sb5. The Lys-54-Glu-61 fragment of Sb5 coincided with the sequence of Sa1. Furthermore, the sequence analysis of Sa5 and Sb5 confirmed the only replacement: the C-terminal residue Glu-53 of Sa5 was replaced by Asp in Sb5 (Fig. 3).

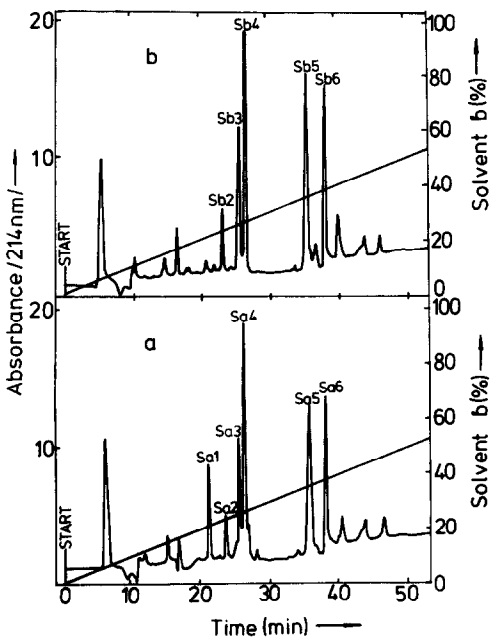

Fig. 5. HPLC of *Staphylococcus aureus* hydrolysates of vinylpyridylated PC*a* (a) and PC*b* (b). 0.1 mg was chromatographied in the conditions as in Fig. 4.

4. DISCUSSION

Parsley plastocyanin is of particular interest because its primary structure sufficiently differs from that of other higher plants. These differences find expression in: (i) the deletions of two residues at positions 57 and 58; (ii) the changes in the charged residues at several locations on the protein surface; and (iii) the addition of an extra tyrosine at residue 62 (Ambler, R.P. and Sykes A.G., unpublished work cited in [8]). Recently, however, similar 'strangeness' was also found in plastocyanin from barley [22], rice [14] and carrot (Shoji et al., cited in [14]). In this respect the 'strange' plastocyanins surprisingly resemble the investigated green alga plastocyanins more closely than those of the higher plants. The IEF data, implying a molecular heterogeneity, have to be considered as an additional challenge for further investigations of parsley plastocyanin. The assumption for diamorphism was confirmed by the last steps of the protein purification.

The procedure for isolation of poplar PC*a* and PC*b* [19,20] was found to be unsuccessful in the case of parsley. The IEF analysis showed the PC-molecular heterogeneity both in PC(II) and PC(I) (Fig. 1b). The separation of parsley PC*a* and PC*b* was performed by chromatofocusing of PC(II) on PBE 94. The small difference of their pI-values ($\Delta pI = 0.02$) was found to be sufficient for separation of the iso-proteins by linear pH 5.5–4.0 gradient elution with Polybuffer 74 (Fig. 2a).

The sequence analysis of the whole iso-proteins as well as of the tryptic and staphylococcal protease peptides revealed that the PC*a*-sequence was identical with the parsley plastocyanin primary structure, previously investigated [8]. PC*b* differed only at 53 position where Glu in PC*a* was replaced by Asp (Fig. 3).

Six major tryptic peptides, T1, T2, T3, T5, T6 and T7, were obtained from PC*a* and PC*b* (Figs. 3 and 4). T4 and T8 were probably results of unspecific trypsin hydrolysis of peptide bonds close to hydrophobic (Leu-63 about T4) or aromatic (Phe-19 about T8) amino acid residues. The replacement Glu-53 → Asp-53 is charge-equivalent and does not affect the chromatographic mobilities of T6. The trypsin peptide maps of PC*a* and PC*b* are practically indiscernible (Fig. 4).

The *Staphylococcus aureus* hydrolysis of PC*a* and PC*b*, however, resulted in two different peptide maps (Fig. 5). The short Sa1 peptide was found to be only PC*a*-derivative (Fig. 5a). This fact of principle could be explained by the already identified Glu-53 → Asp-53 replacement. In a weakly alkaline medium (pH 7.8) *Staphylococcus aureus* specifically hydrolyzes Glu-X peptide bond [24]. In the same conditions the polypeptide chain of PC*b* is not liable to a hydrolysis at

Asp-53-Lys-54 and Sb5 peptide remains six residues longer (Fig. 3). The chromatographic mobilities of Sa5 and Sb5 are practically equal probably due to the additional charge at Glu-61 in Sb5, 'neutralizing' the hydrophobic pattern of Ile-55-Glu-61 (Fig. 5). The consecutive arrangement of four negative charges was probably obstructive to an enzymatic hydrolysis at Asp-42-Glu-45. In addition, the availability of Pro and/or bulky vinylpyridylated Cys-84 nearby probably hinders the hydrolysis of the Glu-85-Pro-86 bond (Fig. 3).

The combination of trypsin and *Staphylococcus aureus* hydrolysis was found to be a useful approach for sequence analysis of parsley plastocyanins. The same method has been applied for determination of *Ulva arasakii* plastocyanin primary structure [13].

Parsley is the second plant where two iso-plastocyanins have been isolated and analyzed in their primary structures. It is unlikely, however, that PC*a* and PC*b* are distinguishable in the amino acid sequences only. Further comparative studies of PC*a*/PC*b* redox characteristics will probably elucidate the physiological importance of the plastocyanin polymorphism.

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